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ACCELERATION OF DETERIORATION OF BACTERIAL CELLS UNDER INFLUENCE OF CERTAIN SUBSTANCES

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/Figures 1-49 referred to in the text have not been reproduced. However, translated captions for these figures are appended to this report.

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Bacteriolysis, the decomposition of bacterial cells, can be induced by a very considerable number of substances; for example, see Gamaleya's list (1). These substances vary greatly in their nature and include substances produced by animals, plants, and microorganisms. The list of these substances is great and has been growing rapidly in recent years due to developments in the field of antibiotics. Bacteriolyzing agents include such diverse substances as bacteriophages and formalin. Among these agents one can find very simple chemical substances as well as high-molecular proteins whose structures have yet to be discovered.

The range of action, i.e., the spectrum of action of lytic agents, varies considerably. Some substances have a very wide spectrum and are capable of bacteriolyzing a great number of types of microorganisms belonging to phylogenetically widely diverse classification groups. Other agents are characterized by a narrow selectivity and a strict specificity of their action, being incapable of producing lysis in all members of a species of microbes. Further differences among bacteriolyzing substances show up in their ability to produce lysis in living or dead cells. In the case of agents capable of producing dissolution only in living cells, it should be noted that whether or not lysis takes place depends on the physiological condition of the cell, i.e., whether the cell is in a resting stage or in the process of propagation.

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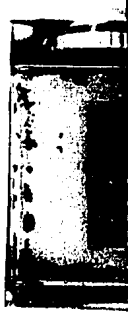


The phenomenon of bacteriolysis is extremely widespread and apparently, all microorganisms are susceptible to the influence of one or another of the bacteriolyzing agents. Taking into account the multiplicity of bacteriolytic agents and the individual peculiarities of the microorganisms, one may assume that lytic agents exert their effects on different parts of the cells, that they affect vital processes of the cells in different ways, and that the chemical reactions involved are varied, despite the fact that the end results of lysis are surprisingly similar in their outward manifestations. Liquid media, murky from the suspension of hundreds of millions, or billions, of living or dead bacteria, become completely clear following the "dissolution" of the microbe bodies, while on solid media dense colonies of microorganisms, which frequently attain large size, gradually fade and disappear, exposing the surface of the medium.

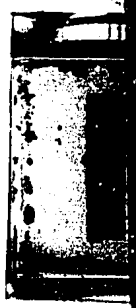
Under an ordinary microscope it is difficult to distinguish the peculiarities of the lytic process which depend on the characteristics of the species involved or on the nature of the lytic agent. First, all that can be seen under maximum magnification of the optical microscope, in the typical process of lysis, is a slight enlargement of the size of the cell after it has come into contact with the bacteriolyzing substance; next, a change in the refraction of the plasma; then, decomposition of the cell body into granules; and finally, "dissolution" of the granules.

In view of the size of bacterial cells, it could be expected that the use of the electron microscope (which possesses a far greater power of magnification than the best models of optical microscopes) would disclose new aspects of the phenomenon of bacteriolysis which were previously concealed from investigators. In the first place, it was interesting to find out whether, with the aid of the electron microscope, it would be possible to detect any specific peculiarities in the manifestation of the process of lysis by comparing the effects of various lytic agents on the same type of microbe.

After a certain amount of search, we succeeded in isolating from the soil a motile bacillus which was sensitive to the action of a whole series of lytic substances and which, consequently, constituted a convenient subject for comparative experimental research. On the basis of its morphological and cultural characteristics and its physiological properties, this bacillus can be considered an atypical form of Bacillus mycoides Flügge.

A necessary prerequisite for examination of biological objects by the electron microscope is the cleansing of these objects from the admixtures of the components of the medium and the products of cellular metabolism and decomposition, since during desiccation of the preparation these impurities may obscure the object itself. A preliminary cleansing is an absolute necessity in preparing materials from bacterial cultures in liquid protein media or saline media. If a drop of such a medium is place on the "object slide" of the electron microscope (a thin collodion film stretched on a wire screen), the examination of the structure of bacterial cells by the electron microscope may be handicapped or made impossible due to the fact that upon desiccation of the drop, to a greater or lesser extent, the components of the broth and salt will conceal significant details of the morphology and cytology of bacteria.

To avoid this, a method of preparation of biological materials for electron microscopy was worked out which permitted cleansing of the object without any injury to the object itself (9). The principle involved in this method, which we called the method of "drop dialysis," consisted in application of the supporting collodion film, used for the preparation of the "object slide" in electron microscopy, as a dialyzing membrane.



Autolysis of Bacterial Cells After Completion of Ontogenesis

Since all of the investigations of the effect of various substances on the isolated, sporulating bacillus were carried out with the aid of the electron microscope, it was first of all necessary to determine whether or not the electron microscopic pictures of the various stages of development of this bacillus were affected by complicating factors of one sort or another. It should be noted that in preparations from cultures of a certain age it is possible to find cells in various stages of ontogenesis. However, if the majority of cells in a preparation had a similar appearance in the field of vision of the electron microscope, then the characteristics of these cells were accepted as typical for cultures of that age, despite the presence of cells in other developmental stages. Special attention is drawn to this fact because, in contrast with optical microscopes (whose field of vision may contain many cells), the screen of the electron microscope accomodates only one cell at a time. Consequently, in electron microscopic observations many preparations must be examined to be assured that the resultant photograph does not reflect a rare or atypical case, as has been known to happen in certain works in electron microscopy. The photographs presented by us record only those pictures which were found to be typical for cultures of a certain age.

The cells in preparations from young (18-24 hr) bouillon or agar cultures absorb electrons with approximately equal sharpness and, consequently, appear uniformly black (Figure 1). They are dense, clearly delineated and are in the stage of active reproduction.

Numerous, repeated observations leave no doubt that the cells presented in Figure 1 are typical of young cultures. Individual variability of the cells shows up during the early stages of the culture. In preparations of 24-hr bouillon cultures, bacilli can be found passing through their life cycle at a somewhat faster rate than the general mass of the cells (Figure 2). In these cells it is possible to observe the beginning of the process of compression of the protoplasm and its polar (axial) separation prior to spore formation. Due to the withdrawal of the protoplasm (which due to its mass and density absorbs electrons), from the cell wall, the latter becomes distinctly discernable as an uncollapsed sac which retains its cellular shape.

The next stage is that of separation of that part of the cell contents which is used for spore production (Figure 3). The rest of the cell contents, the lesser part, gradually becomes transparent (becoming more and more penetrable to electrons), and finally undergoes complete autolysis. As a consequence, the cell becomes empty except in the area where the spore resides (Figure 4).

Apparently the cell retains its turgor until the spore is completely formed. This conclusion can be drawn from the fact that despite the transparency of nearly half the cell (Figure 3), its form remains the same. The process of sporulation is concluded with the collapse of the cell wall (Figure 5). The whole cycle of spore formation of the majority of cells in agar cultures is completed on the 4th day.

The comparison of these electron microscopic observations with our observations of other sporiferous organisms (3), as well as data of other authors, does not reveal any discrepancies. In its general outline, the outward manifestations of cytomorphological changes in the cells during spore formation are identical in many representatives of the sporiferous group of bacteria.

However, quite a number of cells in the cultures of the isolated sporiferous bacillus do not commence sporogenesis. Part of the cells follow a different path of development, but at the beginning of their individual development even these are indistinguishable from cells which eventually develop spores. They

appear to the observer as uniformly black as the spore-producing ones. Later it is possible to see in them signs of separation of their contents, just as in spore formation. But if observations of these cells are continued, the picture changes drastically. The protoplasm becomes fragmented (Figure 6). The fragmented parts are at first dense (strongly absorbing electrons); then they gradually diminish in size and form entities separate from each other (Figure 7). Then more and more new areas within the cell become electronically transparent. Further development of this process of autolysis leads to a complete substitution of compounds of high-molecular weight with low-molecular compounds which are penetrable to electronic rays due to their high degree of dispersion. This process, in its outward manifestation, ends by a complete emptying of the cell, which retains only the cell wall (Figures 8, 9, and 10).

It should be noted that the cell wall is retained for a long time, surviving the process of the developing autolytic decomposition of the cell contents. In very old cultures, one can still encounter cells which have the appearance of empty, transparent cases with a clearly observable, uncollapsed cell wall.

Thus, after the cells cease to multiply, the changes of the protoplasmic colloids, due to age, show up differently depending upon whether or not the cell is developing in the direction of sporogenesis. In the first instance, that part of the plasma content of the cell which forms the spore is preserved while other elements of the protoplasm undergo autolysis. The electron microscopic picture of this process is indistinguishable from the picture of the autolytic decomposition of the entire colloidal mass of those cells which do not form spores.

In both cases, the process develops along lines of disintegration of the protoplasmic elements and of diminution of their mass and density due to the conversion of compounds of high-molecular weight to those of low-molecular weight. This is indicated in electron microscopic observations by the gradual increase in penetration by electrons, first of separate sections of the plasma, and finally of the entire cell. With the exception of the spore, the cell becomes more and more transparent and finally completely so, leaving only the cell wall visible.

In cells which do not form spores the wall remains uncollapsed for a long time which permits us to believe that the "empty" cells are filled with fluid which is transparent to electrons and which balances the pressure of the medium. In cells which have undergone the cycle of sporulation, the cell wall is somewhat disturbed, but this wall is found in a collapsed state on the spore strips.

Phenomena of Autolysis of Bacterial Cells Due to Action of Certain Antiseptics

On beef-peptone media the changes due to age usually set in, in the bulk of the cells of 3-4 day old cultures, after the process of active multiplication is completed. At that time, with the aid of an electron microscope, it is possible to detect with a great degree of reliability, the nonsporulating bacilli by indications of the commencement of the autolytic decomposition of the components of the protoplasm. These appearances of autolysis increase with time and in 5-6 day cultures up to 8-9% of the cells show the cytological transformation so clearly that it is possible to conclude that the process of internal disintegration of the cells is near completion.

However, the process of autolytic transformation of the isolated sporiferous bacillus can be observed considerably earlier if it is subjected to the action of certain substances such as 96% ethyl alcohol, chloroform, or toluol. In the performed experiments, cells from a 24-hr agar culture were spread in a thin film on the bottom of a Petri dish and then 96% alcohol, chloroform, or toluol was poured into the dish. An hour later the liquid was poured off, and after

its residue had fully evaporated, the entire mass of cells was suspended in nutrient broth. After 24 hr, when the cells (which had previously been subjected to the action of alcohol, chloroform, or toluol) were removed from the broth, they clearly displayed the changes which have been described above for nonsporulating bacilli from 4-6 day cultures (Figures 11 and 12).

Despite the differences in cytological reactions due to the individual peculiarities of the cells, it seems obvious that the posthumous changes in the cells, after their exposure to one or another of the antiseptic substances, show up in the form of a well-advanced process of dispersion of the protoplasm, and conversion of complex protoplasmic components into components of low atomic weight. These preparations contained cells representing all stages of autolytic disintegration of the protoplasm, and it was difficult to detect any features which would distinguish them, under the electron microscope, from preparations from old cultures

Although the cells remained in the broth only 24 hr after being killed by the indicated antiseptics, here, as in old cultures of the sporiferous bacillus, it was possible to observe fragmentation of the protoplasm, shrinkage, dissolution of the separate fragments, and finally the almost complete transparency of the cell in which only tiny areas remained which were still capable of partially absorbing electrons. But even in these cases, with brief exposure to alcohol, chloroform, or toluol (under prolonged exposure to these substances lysis of the entire cell may occur), the cell wall was not destroyed. The wall shows up clearly in the "empty" cells in the form of a transparent sac which retains its cellular shape. Such photographs show clearly the invagination of the cell wall at the poles of the bacilli.

Our investigations create the impression that the process of autolysis of the spore-bearing bacillus proceeds more quickly after the bacillus is killed by applied antiseptic substances, than in aging colonies where the corresponding cytological changes proceed at a different rate. It is possible that ethyl alcohol, chloroform, or toluol tends to activate in the dead cells enzymatic hydrolysis reactions which are the basis of autolytic decomposition of protoplasm.

That the observed process of autolysis is based upon the activities of intracellular enzymes can be demonstrated by the following experiment. A 24-hr bouillon culture of the isolated sporiferous bacillus was heated at 80° C for a period of 30 min; then the cells were allowed to remain in the same medium under a layer of toluol for 5 days. Part of the same bouillon culture served as a control. It was likewise kept for 5 days under a layer of toluol but without having been subjected to heating. The comparison of photographs shows that the cells of the unheated broth culture (Figure 13), after spending 5 days under a layer of toluol, had undergone considerable change (Figure 14), while cells which had been heated to 80 degrees for a 30-min period (Figure 15) showed no change after spending 5 days under similar conditions. After the inactivation of cellular enzymes by heat, autolysis does not take place.

Consequently, comparative electron microscopic observations demonstrate that in typical cases the process of autolysis of the sporiferous bacillus can be described (2, 7) as an enzymatic process of disintegration of cellular components (without breaking or collapsing of the cell wall) which creates the effect of completely voiding the cell and leaving the integument in the form of an uncollapsed sac enclosing transparent contents.

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Autolysis and Lysis of Bacterial Cells Under Effect of Gramicidin

It should be noted that the type of posthumous cytological changes in bacterial cells described above does not constitute a unique case. While studying, by means of the electron microscope, the effects of various doses of gramicidin (8) on the isolated sporiferous bacillus, we became convinced that the process of disintegration of bacterial cells can manifest itself in various forms.

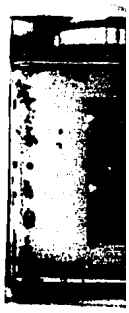
It has been established that gramicidin, as well as other antibiotics, is capable of affecting microbe cells in various ways. Antibiotics are capable of producing bacteristatic, bactericidal, or bacteriolytic effects depending on the species and on the individual sensitivity of the microorganism, on its physiological state at the moment of exposure, on the concentration of the antibiotic, on the length of exposure to action, etc. Consequently, we subjected cultures of the sporiferous bacillus to the effects of increasing concentrations of gramicidin; we varied the lengths of exposure to different dosages of gramicidin; we changed media in which the reaction took place, and we substituted physiological saline solution for beef-peptone broth to be able to observe the reactions of the cells under conditions unfavorable for their multiplication.

It is possible to judge as to whether cells were killed by gramicidin on the basis of cytomorphological changes which commence comparatively soon after cell death. Such cells show up clearly from 3 to 6 hr after exposure to gramicidin before cells which are resistant to gramicidin begin multiplying in large numbers. Under phase-contrast microscope the dead cells stand out by their noticeably translucent protoplasm, while in cases where the process of autolysis is further advanced, protoplasm remains only in separate areas or granules within the cell.

The process of autolytic degeneration of the plasma content of dead cells can be observed with particular clarity on the screen of the electron microscope. With a 1.0 γ/ml concentration of gramicidin, 17% of the cells showed transformation 6 hr after the beginning of the experiment. Among cells which appeared to be in various stages of autolysis within an intact cell wall (Figure 17), it was possible to find cells whose cell walls were broken (Figure 18). This type of cytomorphological change, characterized by broken cell walls (regardless of whether the cell wall was injured before any visible changes in the plasma were observed, or whether injuries occurred at some stage of the autolytic degeneration of the cellular protoplasm), is regarded by us as lysis of the cells.

The increase of concentration of gramicidin to 1.5 γ/ml of broth causes the death of a considerable part of the cells. This is indicated by the presence of a large percentage of bacilli in various stages of autolysis after 6-, 9-, and 24-hr periods of exposure to the action of gramicidin (Figures 19 and 20). As the periods of exposure are increased, the number of cells which have undergone lysis increases perceptably (Figure 21): from 4% (after 1.5- to 3-hr exposure to the action of 1.5 γ/ml of broth) to 32% (after a 24-hr exposure).

With gramicidin concentrations of 2.5 γ/ml of broth, 1.5 hr are sufficient to find 60% of the bacilli in early stages of autolytic decomposition of their protoplasm (Figure 22), while with dosages of 5.0, 10.0, and 25.0 γ/ml, 100% of the cells were autolyzed. After 3 hr of exposure to the effects of gramicidin, among bacilli where disintegration of protoplasm takes place within an intact cell wall (Figure 23), one begins to find cells which had undergone lysis, i.e., cells with broken cell walls (Figure 24). After 6-, 9-, and 24-hr exposures, from 19% to 59% of the cells undergo lysis (Figure 25).



The rest of the bacilli remain with their contents decomposed but with their cell walls intact. Their numbers remain great even after 24-hr exposures to the action of gramicidin in concentrations of 2.5, 5.0, 10.0, and 25.0γ/ml of broth (Figure 26). Krupin (10), in this work on Bacillus mesentericus, likewise notes that a large number of cells did not undergo complete disintegration even under prolonged exposure to gramicidin.

When physiological saline solution was used instead of bouillon, there was a noticeable acceleration of the process of cell destruction from effects of gramicidin. Under these conditions, additions of gramicidin in dosages of 0.5 and 1.0 γ/ml revealed, under the electron microscope, an earlier appearance of autolysis in the cells and at the same time showed a higher percentage of such cells after all exposures to the indicated dosages of gramicidin. For example, after a 1.5-hr exposure to 0.5 γ/ml, 25% of the cells exhibited characteristic cytological changes of the autolytic type (Figure 27), and after a 3-hr exposure, 50% (Figure 28) With dosages of 1.0 γ/ml 50% of the cells showed autolysis after a 1.5-hr exposure (Figure 29), and 80% after 3 hr (Figure 30). An increase in dosage up to 1.5 γ/ml resulted in autolysis of 85% of the cells (Figure 31) after a 1.5-hr exposure to this concentration of gramicidin, etc.

As far as production of lysis in cells is concerned in experiments using gramicidin in physiological saline solution, it must be admitted that quantitative analysis fails to show any significant differences from results obtained in experiments using broth. The time of appearance of the cells undergoing lysis approximately corresponds for physiological saline and broth, as do the percentages after the corresponding exposures to the action of gramicidin.

The performed electron microscopic investigations indicate that the bactericidal action of gramicidin in concentrations which do not exhibit hydrolytic activity of bacterial ferments, produce within a short period an autolytic disintegration of the protoplasm This process goes on during the first hours of exposure to the antibiotic even in concentrations up to 10.0 and 25.0 γ/m. without destruction of the cell wall, i.e., autolytically. After a sufficiently prolonged exposure to the effects of gramicidin, the process develops in the direction of total disintegration of the cell, as a result of breaks in the cell wall. At these stages of its development, such a type of posthumous cytological transformations can be considered as lysis of the cells.

The moment of the involvement of the cell wall in the process of autoenzymatic decomposition of the components of the cell, is determined by the individual peculiarities of the cell. On the one hand, cells undergoing lysis may be encountered (among a majority which have managed to maintain their viability), even after short exposures to weak doses of gramicidin, and on the other hand, 24-hr exposures to such concentrations of gramicidin as 10 and 25 γ/ml do not produce lysis in all cells; approximately half of the cells conclude the process of posthumous decomposition of the plasma with the cell wall intact, i.e., they follow the autolytic type of disintegration.

Lysis, like autolysis, is determined by the activity of the internal enzymes of the cell. The chief external feature which distinguishes lysis from autolysis the involvement of the cell wall in the process of decomposition of the cell contents. This is perhaps due to the changes produced in the cell wall by the influence of gramicidin or other agents. We also observed lysis of the isolated sporiferous bacillus after prolonged (5-13 days) exposures to such substances as toluol and chloroform, which in short periods of action produce only autolysis of the cells. The destruction of the cell wall under the influence of gramicidin can take place at various stages of posthumous changes in the cellular protoplasm, beginning with the first stages of autolysis and ending with the final stage of complete decomposition of the protoplasm.

Inactivation of Enzymes of Bacterial Cell Under Influence of Large Doses of Gramicidin

Posthumous cytological transformations take place, in the sporiferous bacillus, chiefly inside the cell wall which is preserved intact for a long time, i.e., the changes are typically autolytic, not only in dead cells which have completed their ontogenetic cycle, or those which have been killed by the action of chloroform, toluol, or 96% alcohol, but also in cells which have been exposed to the bactericidal action of gramicidin. Comparative electron microscopic observations of cells from old cultures of the sporiferous bacillus, leave the impression that the cell wall is one of the most stable components of the cell during the latter's autolytic disintegration.

Apparently, only after the cell wall undergoes a corresponding transformation, due to the influence of specific substances which affect it, do we obtain a condition which can be considered as lysis, i.e., a partial or complete destruction of the cell wall, which results in an abrupt change in the configuration of the cell, while in autolysis (even in its extreme stages) the cell retains its shape completely

It should be noted that in experiments with gramicidin, the term "autolysis" cannot be used in strict contrast with the term "lysis" since the lysis of a cell under the influence of gramicidin is actually its autolysis, but an autolysis which involves the destruction of the cell wall. The process is specifically autolytic in that the destruction of the cellular components, including the cell wall, is effected by internal enzymes of the cell. Gramicidin brings out "the innate tendency of the cell toward lysis" (4). It is possible that autolysis, in this case, is preceded by injury to certain cell structures which affects the enzymatic activity of a hydrolyzing nature (11, 13, 14, 15, and 16).

It should be easy to understand, therefore, with what interest we watched, in experiments with gramicidin, for results of action of such concentrations of gramicidin, which, having paralyzed the activity of bacterial enzymes, should have, in our opinion, produced a fixing effect.

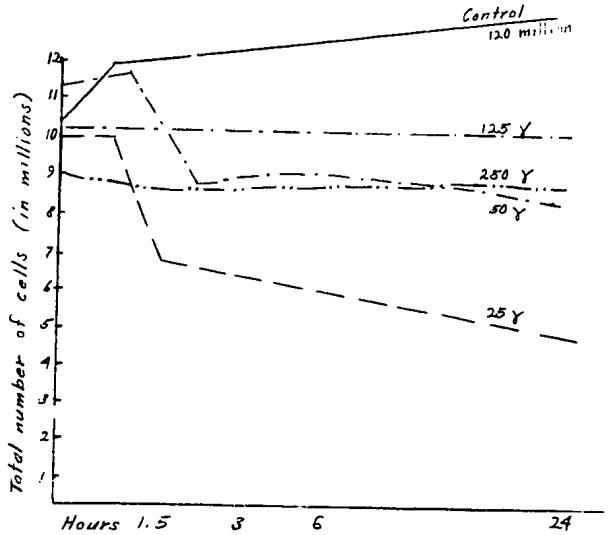


Figure 50. Effects of very high concentrations of gramicidin in broth, on total number of cells of Bacillus mycoides with varied lengths of exposure.

In Figure 50, data showing the determination of the total number of cells with various lengths of exposure to the action of gramicidin in doses of 25, 50, 125, and 250 γ/ml of broth (as well as the control) are presented in form of line graphs. An examination of this graph indicates that the bacteriolyzing effect of this antibiotic can be easily neutralized by using the substance in high concentrations.

The paradoxical aspect of this phenomenon lies in its apparent contradiction of the statements found in literature, to the effect that, as concentrations of certain antibiotics are increased, their bactericidal action is supplanted by a bacteriolytic one. The explanation of this phenomenon lies in the following: it is sufficient to induce chemical inactivation of bacterial enzymes by applying antibiotics in dosages of 125 or 250 γ/ml of broth to inhibit bacteriolysis completely (as concentrations of only 25 or 50 γ/ml fail to do), and to assure that the number of cells does not diminish in 24-hr period. It is interesting that doses of even 50 γ/ml inactivate the enzymes in a considerable number of cells. Only about 20% undergo lysis; the rest do not decompose. When a 25 γ/ml dose of gramicidin is used, the number of cells is reduced to one third as a result of bacteriolysis.

By means of electron microscopic investigations it is possible to establish that cells which are killed by large doses of gramicidin not only fail to undergo lysis, but that they are not even autolyzed. Instead, a process of abrupt concentration and coagulation of the plasma content takes place within the cells. This causes the protoplasm to become shrunken and condensed. The protoplasm withdraws from the walls, chiefly along the longitudinal axis of the cell, appearing intensely black on the screen of the electron microscope; much blacker than the cells of the control sample.

The phenomenon of coagulation of protoplasm shows up with particular clarity in comparisons of electron microscopic pictures of the effects of various dosages of gramicidin. In contrast with the normal, multiplying cells of the control sample (Figure 32), cells which had been exposed to the action of gramicidin (25 γ/ml of broth) for 1.5-hr clearly show changes of the autolytic type (Figure 33). When exposures to this dosage are prolonged, we find evidence of lysis in addition to autolysis (Figure 34). When concentrations of gramicidin are increased to 50 γ/ml of broth, while one still finds many examples of lysis and autolysis (though in smaller numbers), there appear cells whose cytological changes approach a coagulative-type protoplasmic change.

When concentrations are increased to 125 or 250 γ/ml of broth, the electron microscopic picture (after 1.5-hr to 3 of exposure) clearly shows coagulation of the majority of the cells (Figures 35, 36, and 37). With the aid of phase-contrast microscopy (Figures 38 and 39) it is possible to see that the coagulative process is preceded first by a partial condensation of the protoplasm and sometimes by appearance of large granules before complete contraction of the cell takes place.

In this process of coagulation of the protoplasm, the cell wall remains intact and easily separates from the coagulated mass under centrifugation (Figures 36 and 37).

The inactivation of cellular enzymes by such doses of gramicidin as 125 γ/ml and especially 250 γ/ml results in the fact that the coagulated mass of cell contents (drastically changed by the effects of these doses of gramicidin) as well as the cell wall remain preserved for a long time without undergoing autolytic decomposition. If the disintegrative processes of the autolytic type which take place within the cell wall, or those accompanied by a breakdown of the wall, as happens in lysis, indicate the tendency and activity of enzymatic reactions in the cell after its death, then the posthumous cytological changes of the coagulative type indicates a complete paralysis of the enzymatic apparatus of the cell.

All three types of posthumous cytological changes which result from the action of gramicidin--autolysis, lysis, and coagulation -- are simply visible signs of internal cellular reactions to various exposures to, and various doses of, gramicidin.

Catalysis of Enzymatic Reactions of Autolysis of Bacterial Cells

The comparison of the electron microscopic pictures of the autolytic disintegration of the protoplasm of cells from old cultures of our sporiferous bacillus, and from young cultures where cells have been killed by short exposures to chloroform, toluol, or 96% alcohol, or by doses of gramicidin (which were harmless to the activity of the cellular enzymes), seems to indicate that the various differences (due to differences in the nature of the bactericidal agent) in the reactions which bring on the death of the cells, disappear, being invalidated by the disintegrative reactions of the cell after its death. This creates the impression that the posthumous enzymatic reactions within the cells follow completely identical courses regardless of whether death occurred as a result of the natural completion of the entogenetic cycle, or whether it was induced by the action of chloroform, toluol, alcohol, or gramicidin

However, this impression which is based on the superficial resemblance of the electron microscopic pictures of cellular autolysis, is dispelled by a careful comparative analysis of the data of bacteriological and electron microscopic investigations The specific influence of the factor which induces death of the bacterial cell can be observed even in the posthumous phenomena which take place within the cell. The specificity of this influence may be deduced from the different reaction rates of cellular autolysis which, apparently, depend on the methods by which the bactericidal agents in question activate the enzymatic reactions of the hydrolysis of the bacterial protoplasm

It has been previously pointed out that signs of autolysis in the majority of the bacilli, which had not undergone sporogenesis, can be detected in 3-4 day old cultures In 5- or 6—day broth and agar cultures of the isolated sporiferous bacillus, the bulk of the cells exhibit signs of being in the last stages of internal decomposition It has also been shown that when cells of one-day old cultures of the sporiferous bacillus are subjected to the action of chloroform, toluol, or 96% alcohol for an hour and are then placed into nutrient broth for 24-hr it is possible (by means of electron microscopy) to observe in them marked stages of autolysis, comparable to those which are found in the majority of the cells of broth cultures only after 4-6 days

This phenomenon could not be overlooked It suggested the idea that since conditions under which the activity of cellular enzymes took place were identical in relation to temperature and pH, that the difference in reaction rates of enzymatic decomposition of the cell contents was due to the difference in the chemical nature of the activating agent

To be able to arrive at a more complete understanding of the speed of onset of autolysis, after short exposures to the indicated antiseptics, the following experiment was devised

As in the previous experiment, cells of 24-hr agar cultures were spread in a thin layer in the bottoms of Petri dishes The experimental dishes were then filled with 96% alcohol, while the control dishes were filled with broth. After an hour the liquids were drained off, smears of the cells were dried in air, and then part of the cells were suspended in distilled water (to be able to study their condition directly, after their exposure to alcohol); the other part was transferred to nutrient broth After 1.5-, 3-, 6-, 9-, and 24-hr periods in the bouillon, total cell counts were made, followed by plating out on beef-peptone agar to determine the number of live cells in the experimental and control samples.

Simultaneously, cells from the bouillon suspension (those which had been exposed to alcohol), and from the bouillon culture (the control, which had been exposed to bouillon) were settled out by means of centrifugation at 9,000 rpm. The settled out cells were then transferred to drops of distilled water, placed on the "object slide" of the electron microscope and examined.

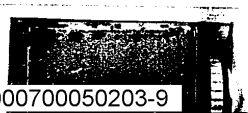
Table 1 indicates that after 24-hr in nutrient broth (after having been exposed to alcohol) the total number of cells remained practically the same.

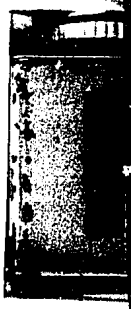
/See Table 1 on the following page./

Table 1. Time of Appearance of Autolysis in Cells of Bacterial Populations Subjected to Action of 96% Alcohol

Periods of Observation (in hr)	Control			After Exposure to Alcohol		
	Total No of Cells	No of Colonies	Percent of Autolyzed Cells	Total No of Cells	No of Colonies	Percent of Autolyzed Cells
1	7,800,000	540,000	0	5,000,000	0	0
1.5	14,600,000	800,000	0	5,200,000	0	0
3	20,000,000	2,720,000	0	5,700,000	0	10
6	40,000,000	25,600,000	0	5,000,000	0	50
9	61,200,000	64,000,000	0	4,600,000	0	100
24	132,000,000	solid growth	0	3,800,000	10	100

STAT





However, as the seeding out on beef-peptone agar has indicated, after an hour of exposure to 96% ethyl alcohol, all the cells were dead. After the remainder of the seeded material has been in bouillon 24-hr some of the spores may grow. By contrast with the results of the control sample, however, the ten colonies which multiplied only underscore the effectiveness of alcohol as a bactericidal agent.

Observations with the aid of the electron microscope indicate that immediately after exposure to alcohol (Figure 40), and likewise after 1.5-hr of remaining in the bouillon (Figure 41), the cells do not differ in appearance from those of the control sample (Figure 42). After exposure to alcohol, bacilli showing signs of autolysis (Figure 43) begin to appear among the mass of cells after remaining for about 3 hr in the nutrient broth, but their number is small (not more than 10%). Only after 6-hr suspension in bouillon (Figure 44) does the percentage of autolyzed bacilli (which had been exposed to alcohol) reach 50%. Only later, 9-24-hr after transfer to the bouillon, does the entire mass of cells (which had been exposed to alcohol) exhibit autolysis in various stages of its development (Figures 45 and 46), depending on the individual peculiarities of the organisms. During all these periods of time, bacilli from the control sample have a normal appearance under the electron microscope.

The data from this experiment reveal that visible signs of autolysis in the cells begin to show up from 3-6 hr after the cells have been killed by the action by the alcohol. By that time (3-6 hr) half the cells show, under the electron microscope, cytological changes indicating decomposition of their cellular contents.

By comparing the effects of alcohol with those of gramicidin on the same organism, we can see that gramicidin possesses a definite ability to accelerate autolytic reactions in cells.

Table 2. Increase in Number of Autolyzed Cells in Bacterial Population in Relation to Increase of Concentration of Gramicidin

	After 1.5 Hr Exposure to Gramicidin in Bouillon in the Following Concentrations						
	Control	1.0 γ/ml	1.5 γ/ml	2.5 γ/ml	5.0 γ/ml	10.0 γ/ml	25.0 γ/ml
Total no of cells	2,200,000	4,200,000	4,000,000	3,800,000	4,200,000	3,800,000	3,600,000
No of colonies	690,000	580,000	280,000	74,000	0	0	0
Percentage of autolyzed cells	0	3	48	70	100	100	100

By referring to Table 2 we can get some idea of the time it takes, after exposure to gramicidin, for signs of autolytic disintegration of the protoplasmic components to show up. With concentrations of 1.5 γ of the antibiotic per milliliter of broth, 48% of the cells show autolysis after a 1.5-hr exposure. By

comparing the number of colonies obtained with this dose and exposure one comes to the conclusion that more than half of the cells were killed by 1.5-hr contact with a 1.5 γ/ml dose of gramicidin.

Consequently, in the greater part of the cells killed by gramicidin, the actively advancing processes of autolysis can be revealed by the electron microscope much earlier than in those killed by alcohol. By increasing the dosages of gramicidin, and, by the same token, increasing the number of dead cells, it is possible, if gramicidin is used in concentrations of 5.0, 10.0, and 25.0 γ/ml of bouillon, to cause death of all of the cells. Consequently, on the screen of the electron microscope it is possible to observe 100% of the cells undergoing the type of cytological changes which are characteristic of the autolytic process of the breakdown of protoplasm (Figures 47, 48, and 49).

Thus in cells killed by 96% alcohol the process of autolysis shows up in 100% of the cells only after 9 hours (after death), while in cells of the same culture of the sporiferous bacillus killed (during the first minutes) by contact with 25 γ/ml of gramicidin, autolytic decomposition is apparent, under the electron microscope, in 100% of the cells 1.5-hr after their death. Based upon the time required for autolysis to appear in the bulk of the cells, we get the following series. In cultures where cells had completed their cycle of development, without being subjected to any special reagents, autolysis appears after 5 or 6 days (A Manteyfel' [127] observed after the expiration of 24-25 hr of cultivation of acetone-butyric acid bacilli on an alumin medium, that about 50% of the cells underwent autolysis); in cultures killed by alcohol, autolysis appears after 9 hr; in cultures killed by gramicidin, after 1.5-hr.

But since the time of death in aging cultures (those which are completing their life cycles) cannot be established, we cannot know how long after death signs of autolysis appear. Consequently, only the data from experiments of . . . the effects of alcohol and gramicidin on the length of time required for autolysis to appear can be compared.

The comparison of these data suggests that the effect of the bactericidal agent can be revealed even after the death of the cell, specifically by the extent to which it activates the cellular enzymes which participate in the process of autolysis. It is quite probably that the means of activation of enzymatic systems (whose action serves to hydrolyze proteins, fats, carbohydrates, and other components of the cellular protoplasm) differ, depending on the nature of the agent which causes death of the cell. It now becomes quite obvious, as we noted earlier in relation to bacteriophage (5, 6), that a whole series of substances is capable of acting as catalysts in the autolysis of the bacterial cell.

Here we come across a phenomenon that broadens considerably our concepts concerning activators of enzymatic processes. In addition to activators of individual enzymes, or of specific enzymatic systems, it becomes necessary to acknowledge the existence of agents which can simultaneously activate hydrolysis of many cellular enzymes belonging to the most diverse groups. Evidence of this manifests itself externally by the rapid reaction rate of decomposition of the chemically varied substratum which composes the body of the bacterial cell. Observations of the autolytic process due to the exposure to gramicidin brings us to the conclusion that the influence of this antibiotic is not limited to a single cellular enzyme, or even to a group of enzymes belonging to a single class; for example, protease, which clearly effects the onset of autolysis. A definite accelerating effect is also produced on lipase, on enzymes which cause the breakdown of carbohydrates, as well as on other enzymes, the joint activity of which brings about the transformation of the components of protoplasm into soluable compounds capable of being diffused through the cell wall.

It is difficult to say much about the mechanism of this phenomenon. We cannot tell whether the substance which accelerates the process of cellular autolysis has a universal action in relation to the hydrolyzing activity of various cellular enzymes, or whether it activates directly or indirectly, by suppressing the effect of an inhibitor (corresponding to a "release" mechanism) and thus directing the activity of cellular enzymes in the direction of hydrolysis. This is an area for further research. But the very nature of the processes taking place permits us to conclude that a whole series of substances exist which are capable of activating the enzymatic reactions of autolysis in bacterial cells. Such substances can, consequently, be called autolytic catalysts.

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CAPTIONS FOR FIGURES 1-49

Figure 1. Cells fro.. 24-hr culture in beef-peptone bouillon (MPB, meat-peptone bouillon).

Figure 2. Spore-forming cells from 24-hr culture in MPB.

Figure 3. Spore-forming cells from 72-hr culture on beef-peptone agar (MPA, meat-peptone agar).

Figure 4. Spore-forming cells from 72-hr culture on MPA

Figure 5. Spores from 72-hr culture on MPA.

Figure 6. Cells with indications of commencing autolysis from 96-hr culture on MPA.

Figure 7. Cells undergoing autolysis from 9-day culture in MPB.

Figure 8. Cells undergoing autolysis from 6-day culture in MPB.

Figure 9. Same as Figure 8

Figure 10. Cells undergoing autolysis from 10-day culture on MPA.

Figures 11 and 12. Cells from 24-hr culture on MPA after exposure to 96% alcohol for one hour followed by 24 hr in MPB

Figure 13. Cells from 24-hr culture in MPB.

Figure 14. Cells undergoing autolysis from a 24-hr culture in MPB, after remaining for 5 days under a layer of toluol.

Figure 15. Cells from a 24-hr culture in MPB heated to 80°C for 30 min.

Figure 16. Cells from 24-hr culture in MPB, heated to 80° C for 30 min and then remaining for 5 days under a layer of toluol.

Figures 17 and 18. Autolysis and lysis of cells in MPB contining 1 Y of gramicidin per ml (6-hr exposure)

Figure 19. Autolyzing cells from MPB containing 1.5 Y of gramicidin per ml (6-hr exposure).

Figure 20. Autolyzing cells from MPB containing 1.5 Y of gramicidin per ml (24-hr exposure).

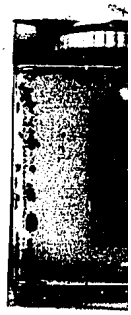

Figure 21. Cells undergoing lysis from MPB containing 1.5 γ of gramicidin per ml (9-hr exposure).

Figure 22. Autolyzing cells from MPB containing 2.5 γ of gramicidin per ml (1.5-hr exposure).

Figure 23. Autolyzing cells from MPB containing 5 γ of gramicidin per ml (3-hr exposure).

Figure 24. Cells undergoing lysis from MPB containing 2.5 γ of gramicidin per ml (3-hr exposure).

Figure 25. Cells undergoing lysis from MPB containing 5 γ of gramicidin per ml (6-hr exposure).

Figure 26. Autolyzing cells from MPB containing 2.5 γ of gramicidin per ml (24-hr exposure).

Figure 27. Autolyzing cells from physiological saline solution containing 0.5 γ of gramicidin per ml (1.5-hr exposure).

Figure 28. Autolyzing cells from physiological saline solution containing 0.5 γ of gramicidin per ml (3-hr exposure).

Figure 29. Autolyzing cells from physiological saline solution containing 1.0 γ of gramicidin per ml (1.5-hr exposure).

Figure 30. Autolyzing cells from physiological saline solution containing 1.0 γ of gramicidin per ml (3-hr exposure).

Figure 31. Autolyzing cells from physiological saline solution containing 1.5 γ of gramicidin per ml (1.5-hr exposure)

Figure 32. Cells from 1.5-hr culture from MPB (control)

Figure 33. Autolyzing cells from MPB containing 25,0 γ of gramicidin per ml (1.5-hr exposure).

Figure 34. Cells undergoing lysis from MPB containing 25.0 γ of gramicidin per ml (3-hr exposure).

Figure 35. Cells with coagulated protoplasm from MPB containing 125 γ of gramicidin per ml (3-hr exposure).

Figure 36. Cells with coagulated protoplasm from MPB containing 250 γ of gramicidin per ml (1.5-hr exposure).

Figure 37. Cells with coagulated protoplasm from MPB containing 250 γ of gramicidin per ml (3-hr exposure).

Figure 38. Cells from 24-hr culture in MPB (control). Phase-contrast microscopic enlargement 1,200 times.

Figure 39. Cells with coagulated contents from MPB containing 125 γ of gramicidin per ml (3-hr exposure). Phase-contrast microscopic view, enlargement 1,200 times.

Figure 40. Cells from 24-hr culture on MPA immediately after a one-hour exposure to 96% ethyl alcohol.

Figure 41. Cells from 24-hr culture on MPA after a 1-hour exposure to the action of 96% alcohol followed by 1.5-hr suspension in bouillon.

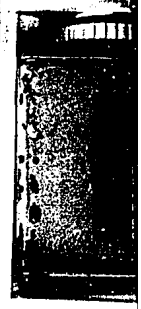

Figure 42. Cells from 24-hr culture on MPA (control).

Figure 43. Cells from 24-hr culture on MPA exposed to 96% ethyl alcohol for one hour followed by a 3-hr suspension in bouillon (initial indications of autolysis).

Figure 44. Cells from 24-hr culture on MPA exposed to 96% ethyl alcohol for one hour followed by 6-hr suspension in bouillon (presence of autolysis).

Figure 45. Cells from 24-hr culture on MPA exposed to 96% ethyl alcohol for one hour followed by 9-hr suspension in bouillon (presence of autolysis).

Figure 46. Cells from 24-hr culture on MPA exposed to 96% ethyl alcohol for one hour followed by 24-hr suspension in bouillon (presence of autolysis)

Figure 47. Autolyzing cells from MPB containing 5.0 γ of gramicidin per ml (1.5-hr exposure).

Figure 48. Autolyzing cells from MPB containing 10.0 γ of gramicidin per ml (1.5-hr exposure).

Figure 49. Autolyzing cells from MPB containing 25.0 γ of gramicidin per ml (1.5-hr exposure).

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